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RESEARCH PAPER

Gender-related differential effect of tachykinin NK_2 receptor-mediated visceral hyperalgesia in guinea pig colon

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BACKGROUND AND PURPOSE

The tachykinin NK_2 receptor antagonist ibodutant is under Phase III clinical investigation to treat female patients with irritable bowel syndrome. The aim of this study was to investigate the NK_2 receptor-related gender specificity in a model of colitis.

EXPERIMENTAL APPROACH

Colitis was induced by rectal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS, 0.5 mL, 30 mg·mL^{-1} in 30% ethanol) in female and male guinea pigs. Electromyographic recording of the responses to colorectal distension (CRD) was made 3 days later. Ibodutant (0.33 , 0.65, 1.9 and 6.5 mg·kg^{-1}) was given s.c., 30 min before CRD. Release of neurokinin A and substance P from isolated mucosal and smooth muscle tissues following treatment with KCl (80 mM) or capsaicin (10 μM) was measured by EIA. Plasma pharmacokinetics of ibodutant following a single s.c. administration (0.73 or 2.1 mg·kg^{-1}) were measured over 24 h.

KEY RESULTS

Ibodutant did not affect abdominal contractions in control animals. After TNBS-induced colitis, ibodutant prevented the increased visceral hypersensitivity to CRD in females, at lower doses than in males. Ibodutant pharmacokinetics did not differ between females and males. Tachykinins release was greater in smooth muscle than in mucosal samples. Capsaicin-stimulated release of tachykinins from inflamed mucosal samples from females was significantly lower than in males.

CONCLUSIONS AND IMPLICATIONS

Ibodutant prevented abdominal nociception in a model of visceral hypersensitivity in guinea pigs with a greater efficacy in females than in males. Our results highlight a gender-related difference in colonic visceral hypersensitivity and mucosal nerve activation.

Abbreviations

IBS, irritable bowel syndrome

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Tables of Links

TARGETS
GPCRs
NK_1 receptor
NK_2 receptor
NK_3 receptor

LIGANDS
Capsaicin
Ibodutant
NKA, neurokinin A
SP, substance P

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorder, characterized by altered bowel habits associated with abdominal discomfort or pain (Camilleri *et al.*, 2012). Colonic pain thresholds to mechanical distension are markedly decreased in IBS patients, indicating that these patients have an altered visceral hypersensitivity, which has been attributed to modifications of the sensory-motor function of the intestine (Mertz, 2002; van Wanrooij *et al.*, 2014).

Tachykinin NK_1, NK_2 and NK_3 receptors are all abundantly expressed in the gastrointestinal tract with similar distribution in humans and guinea pigs (Holzer and Holzer-Petsche, 2001), and tachykinins, which are present at high density in the gastrointestinal tract, are considered as key mediators in the communication between sensory neurons and effector cells, such as smooth muscles, glands and immune cells. Several pieces of evidence have established a link between the tachykinin NK_2 receptor type and IBS (Lecci *et al.*, 2004).

Ibodutant is a non-peptidic compound, exhibiting high-potency and long-lasting selective antagonist activity at the human tachykinin NK_2 receptor, both recombinant and natively expressed in the colon (Meini *et al.*, 2009; Santicioli *et al.*, 2013). It is effective in inhibiting colonic contractions induced by NK_2 receptor agonist stimulation in the anaesthesized guinea pig (Cialdai *et al.*, 2006). Recently, the results of a Phase II clinical study on the efficacy and safety indicated that ibodutant was effective in significantly ameliorating the pain conditions of only female patients with IBS (affected by diarrhoea: IBS-D) (Tack *et al.*, 2013). Although several reports have highlighted the sex-related differences in visceral perception of IBS patients and several attempts in clarifying the role of sex hormones in altering IBS symptoms have been made, the mechanisms involved in this process are still not fully understood (Fillingim *et al.*, 2009; Labus *et al.*, 2013; Meleine and Matricon, 2014).

The aim of this study was to investigate any possible gender-related differences played by tachykinin NK_2 receptors that could account for the observed greater efficacy of ibodutant in female IBS patients, by investigating the effect of ibodutant in a guinea pig model of visceral hyperalgesia induced by inflammation, along with its pharmacokinetic profile. We also studied the release of tachykinins from isolated tissues taken from control and inflamed animals. We selected guinea pigs for this investigation on the basis of the antagonist and affinity profile of ibodutant that had been previously shown to be in the nanomolar range at the human and guinea pig tachykinin NK_2 receptors, but in the micromolar range in rats and mice (Cialdai *et al.*, 2006).

Methods

Animals

All animal care and experimental procedures were approved by the Italian and French Institutional Animal Care and Use Committees, and complied with the European Community guidelines (CEE Directive 86/609). The animal studies follow the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). Male and female adult Dunkin–Hartley guinea pigs (Harlan, France and Italy) 427 ± 28 and 410 ± 34 g (5–6-weeks of age), respectively, were used. Guinea pigs were kept under standard animal housing conditions (12 h light/dark cycle), with food and water *ad libitum*. The hormonal status of the females was not controlled.

Visceral hyperalgesia model

Animal preparation and electromyographic recordings. Animals were anaesthetized by i.p. injection of acepromazine (0.5 $mg{\cdot}kg^{-1}$) and ketamine (100 $mg{\cdot}kg^{-1}$). Three pairs of nichrome wire electrodes were implanted bilaterally into the abdominal external oblique muscle, 2 cm laterally from the midline, just superior to the inguinal ligament. The free ends of electrodes were exteriorized on the back of the neck. Abdominal striated muscle electromyographic (EMG) recordings were performed under anaesthesia and started 5 days after surgery. The electrical activity was recorded with an electromyograph machine (Mini VIII; Alvar, Paris, France) using a paper speed of 4 $cm{\cdot}min^{-1}$, and a short-time constant (0.03 s) to remove low-frequency signals (<3 Hz).

Induction of inflammation. Animals were anaesthetized with i.p. acepromazine (0.5 $mg{\cdot}kg^{-1}$) and ketamine (100 $mg{\cdot}kg^{-1}$). 2,4,6-Trinitrobenzenesulfonic acid (TNBS, 0.5 mL; 30 $mg{\cdot}mL^{-1}$) in 30% (v/v) ethanol was administered into the lumen of the colon through a polyethylene catheter inserted via the rectum to 7 cm from the anus.

Colorectal distension (CRD) procedure. The CRD procedure was carried out 3 days after the instillation of TNBS. Thirty minutes before the CRD procedure, animals were treated s.c. with vehicle (PBS pH 6.4, 10% DMSO) or ibodutant at the doses of 0.3 (only females), 0.65, 1.9 and 6.5 mg·kg^{-1}.

Guinea pigs were pre-anaesthetized by i.p. injection of pentobarbital (9.1 mg·kg^{-1}) and a second dose (5.5 mg·kg^{-1}) after 1 h. The anaesthesia was maintained during all the distension procedure by an s.c. infusion of pentobarbital (1.5 mg·h^{-1}).

The balloon used for CRD procedure consisted of an arterial embolectomy catheter of 2 mm diameter (Fogarty; Edwards Lifesciences, Irvine, USA) with a ligated 4 cm long latex condom balloon, which was inserted into the rectum to 1 cm from the anus, and the catheter was fixed at one foreleg. The balloon was progressively inflated with water, using a 2 mL syringe, by steps of 0.5 mL (from 0 to 2 mL), each inflation step lasting 5 min.

Release experiments

Animals were killed by exposure to CO_2. A segment of distal colon (5–6 cm in length, taken between 2–3 and 8 cm from the anus) was rapidly excised and transferred to a Silgard®-coated Petri dish containing oxygenated (96% O_2 and 4% CO_2) cold Krebs solution (mM composition: NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KCl 4.7 and glucose 11). The colon was opened, by cutting it along its mesenteric border, and pinned flat with the mucosal side up to the Silgard® base. The mucosal and muscle layers were carefully separated under microscope dissection. Histological studies (data not shown) conducted with haematoxylin-eosin. The immunohistochemistry studies using the Protein Gene Peptide 9.5 were performed to count neurons of the submucosa, remaining on the muscle or on the mucosa subdivided colon tissue. The number of stained neurons in the submucosa did not differ in the two preparations, thus excluding a relevant contribution by the submucosal neurons on the observed release. Then, tissues were sliced (thickness 0.4 mm) using a MacIlwain tissue chopper (Campden Instruments, Leicester, UK). Aliquots of tissue slices (muscle 100–150 mg; mucosa 170–230 mg) were transferred into thermostated (37°C) 1 mL Perspex perfusion chambers and superfused, at a flow rate of 0.4 mL min^{-1} oxygenated Krebs solution containing 0.1% BSA and 10 μM thiorphan. After a 60 min stabilization period, superfusates were collected before (basal, 15 min period), and after stimulation with KCl (80 mM) or capsaicin (10 μM) (15 min period). Stimulation with high-potassium Krebs solution was prepared by replacing equimolar amount of NaCl with KCl. Superfusates were collected into polypropylene tubes containing acetic acid (2N final concentration). Samples were extracted by using C18 Sep-Column (Peninsula Laboratories International, San Carlos, CA, USA) according to the manufacturer's instructions. Extracted samples were frozen in liquid N_2, lyophilized and stored at −20°C until assayed for the peptides. For these assays, the samples were reconstituted in assay buffer. Each sample was assessed in duplicate and diluted when necessary. The concentration of substance P (SP) or neurokinin A (NKA) was determined by EIA kits (s-1180 and s-1218; Peninsula Laboratories International). Each sample was measured in duplicate, and the mean value was used for further calculation. Peptide amounts were normalized according to the reconstitution volume and the tissue wet weight.

Pharmacokinetic studies

The plasma pharmacokinetics of ibodutant following s.c. administration of the drug (0.73 or 2.1 mg·kg^{-1}) in male and female guinea pigs was determined. Four different groups of animals were used: each guinea pig received a single dose, followed by blood sampling. On the day before treatment, the right jugular vein was cannulated, under ether anaesthesia, with a silastic® tube (0.635 mm inner diameter (I. D.), 1.1938 mm outer diameter (O. D.)) to permit blood sampling. Blood samples (approximately 0.30 mL each) were collected from all animal groups in tubes containing 20 μL of Eparina Vister® (diluted 1:10 with sterile physiological solution) by means of the cannula inserted in the jugular vein. Blood were drawn at the following time points: predose, 5, 15, 30 min, 1, 2, 4, 8, 24 and 48 h. Blood sample tubes were gently shaken, placed on ice and immediately centrifuged at 2800 × g for 10 min at 4°C. Then, plasma was transferred into a polypropylene tube and stored at −20°C until analysis.

Quantitative analysis of ibodutant plasma content

Plasma content of ibodutant was determined by LC/tandem MS (LC-MS/MS). Samples were first extracted: 0.05 mL of plasma was diluted and acidified with 0.05 mL of 4% phosphoric acid (85%) and spiked with 10 μL of 0.1 μg·mL^{-1} of[D-phenyl-d5-alanine]-ibodutant. The sample was loaded onto a Waters Oasis® μElution Plate cartridge HLB (30 μg) well, which was pre-conditioned with 0.2 mL of methanol and 0.2 mL of HPLC-water. The well was dried out and washed with 0.05 mL of 80/20 (v/v) water/methanol and 0.05 mL of 50/50 (v/v) water/methanol; the test items were eluted in three (0.05 mL) steps with methanol. The eluate was transferred into the autosampler phial, and 10 μL of it was injected into the chromatographic system.

The HPLC-MS/MS system consisted of an LC-20ADXR solvent delivery pumps (Shimadzu, Kyoto, Japan) equipped with an automatic sample injector model SIL-20AXR and a temperature system controller model CTO-20A (Shimadzu), a column KINETEX 2.6 μm C18 2.1 × 50 mm (Phenomenex, Torrance, CA, USA) and a MS/MS detector API 2000 (AB SCIEX; Framingham, MA, USA). The entire system was controlled by the Analyst® software (vers. 1.5.1 AB SCIEX). The mobile phase consisted of gradient A (99/1 v/v water/methanol containing 5 mM ammonium formate and 0.05% v/v formic acid) and gradient B (acetonitrile containing 0.05% v/v formic acid). The flow rate was 0.75 mL·min^{-1} with a split, 0.45 mL·min^{-1} being introduced into the mass detector. Column temperature was kept at 40°C. The following gradients were used: t = 0–3 min, 90% A; t = 3.2–5 min, 50% A; t = 5–6 min, 10% A; t = 6–7 min, 10% A; and t = 7–8 min, 90% A. Column temperature was 30°C. Ibodutant and internal standard were detected by positive electrospray ionization tandem quadrupole mass spectrometer in multiple reaction-monitoring on a API 2000 (AB SCIEX). Gases were fixed as follow: ion source gas 1, 45 pounds per square inch (PSI) (air); ion source gas 2, 60 PSI (air); collision

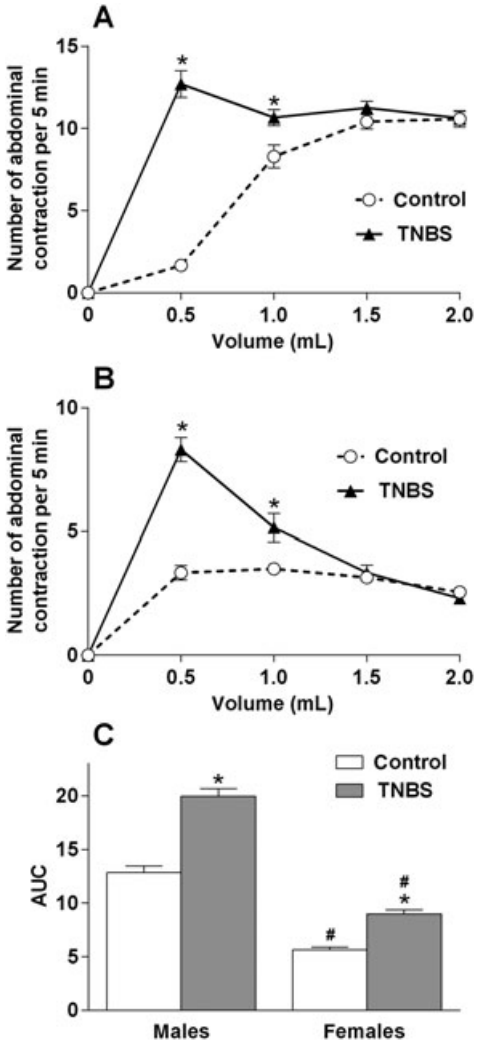

Figure 1

Number of abdominal contractions following CRD in control and TNBS-treated male and female guinea pigs. Data are expressed as number of abdominal contractions measured in 5 min periods or AUC. Data points are the mean ± SEM of 24 (male: A) or 32 (female: B) animals in each group. Data were analysed by two-way ANOVA followed by Bonferroni's multiple comparison test. *$P < 0.05$ versus control group. (C) AUC has been calculated from data presented in A and B. *$P < 0.05$ versus control group; #$P < 0.05$ versus the corresponding male group.

gas, 3 PSI (nitrogen) and curtain gas, 40 PSI (nitrogen). Ionization voltage and ion source temperature were 5600 V and 550°C, respectively. Ibodutant-monitor ions were 645.3 > 175.1 while the internal standard ion were 650.03 > 175.0.

The analytes had the following retention times: 3.8 min for both analyte and internal standard. The linearity of the method (mean $r^2 = 0.9962$) was assessed in the range between 0.02 and 10 ng·0.05 mL^{-1} of matrix. The lower limit of quantification (LLOQ) for ibodutant was 0.4 ng·mL^{-1}. The within-run and between-run precision of the method demonstrated for the LLOQ, and expressed by the coefficient of variation (CV), was ≤9.2%. The within-run and between-run precision of the method demonstrated for the low, medium, and high quality control (QC) samples was ≤11.2% (CV). The within-run and between-run accuracy of the method at the LLOQ, expressed as percent of the nominal value, was between 97% and 106%. The within-run and between-run accuracy of the method at the low, medium and high QC, expressed as percent of the nominal value, was between 99% and 109%. The method was selective, and no evidence of unacceptable carry over was observed. The extraction procedure gave recoveries ≥107% (≤120%).

Data and statistical analysis

These studies complied with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015), except as explained below. All animals were randomized into groups by using GraphPad QuickCalcs available online (http://www.graphpad.com/quickcalcs/randomize1.cfm), and all experiments were performed without knowledge of the treatments (blinding). Data are reported as mean ± SEM or SD when indicated, and n is the number of animals. $P < 0.05$ was taken as the significance level, and *post hoc* tests were run only if the F value deriving from ANOVA achieved the level of statistical significance.

In the visceral hyperalgesia experiments, the abdominal EMG spike bursts corresponding to contractions of the abdomen and observed during each distension level (5 min) were counted and analysed by using two-way ANOVA followed by the multiple comparison *post hoc* Bonferroni test. Data on KCl or capsaicin-induced peptide release from mucosa and colon smooth muscle tissues were corrected for the corresponding previous basal value. Because of the multiple steps in the whole procedure of release experiments from colon specimens, several samples were lost, that is, during the superfusion, some samples occluded the system and the superfusate could not be collected, or an occlusion occurred during the extraction procedure. This explains the different numbers of samples in the results reported. These results were analysed by two-way ANOVA followed by Fisher's least significant difference t-test, using GraphPad PRISM version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

The pharmacokinetic parameters of individual guinea pigs were calculated by fitting concentrations of ibodutant versus time obtained after s.c. administration as a compartment model-independent analysis using Kinetica Version 4.4.1 (Thermo Electron Corporation, Philadelphia, PA, USA). Further, the following pharmacokinetic parameters of individual animals were obtained: maximun concentration (C_{max}) and the area under concentration–time curve from zero (pre-dose time) to the last drug detectable concentration (AUC_{0-t}). The AUC value for each animal was also calculated by using GraphPad Prism.

Materials

Ibodutant (previously named MEN15596, [1-(2-phenyl-1R-{[1-(tetrahydropyran-4-ylmethyl)-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]amide) was synthesized in Lusochimica S.p.A. (Lomagna, Italy). Acepromazine was Calmivet® (Laboratoire Vétoquinol, Lure, France), and ketamine was Imalgene® 1000 (Rhone Mérieux, Lyon, France). Pentobarbital sodium was from Chemische Fabrik Berg

(Bitterfeld-Wolfen, Germany). TNBS solution (1 M in H_2O, Sigma-Aldrich, St. Louis, MO, USA) was freshly diluted as previously indicated. Capsaicin (from Sigma-Aldrich) stock solutions were prepared in ethanol at 10 mM concentration, with further dilution using Krebs solution on the day of experiment. All other compounds used were from Sigma-Aldrich.

Results

Colorectal distension (CRD) in control and TNBS-treated male and female guinea pigs

The CRD procedure increased the occurrence of abdominal contractions, corresponding to a lowering of the nociception threshold, which had a different pattern in control animals. In male guinea pigs, abdominal contractions increased in a volume-related manner (0.5–2.0 mL, Figure 1A), whereas in females, a maximal effect was produced by the lower distension volume (0.5 mL) without any further increase at the higher distension volumes (Figure 1B). Moreover, the CRD applied to animals treated with intrarectal TNBS to induce inflammation 3 days before, produced a 7.6-fold increase of abdominal contractions, compared with control animals at the distension threshold volume (0.5 mL) in males and a 2.5-fold increase in females (Figure 1A and B). In order to quantify the whole stimulus–response curve, the AUC value for each animal was calculated, and these data are presented in Figure 1C. The analysis of data by using two-way ANOVA indicated a response to CRD that significantly depended on both gender and TNBS inflammatory treatment (F_{gender} (1, 108) = 2266, $P < 0.05$; $F_{TNBS\ treatment}$ (1, 108) = 753, $P < 0.05$), apart from indicating a significant interaction between these two paramenters ($F_{interaction}$ (1, 108) = 95.58, $P < 0.05$).

Effect of ibodutant on response to CRD

The s.c. administration of ibodutant 30 min before CRD did not significantly modify the number of abdominal contractions under basal conditions (animals without inflammation) at any of the tested doses, both in female (0.33, 0.65, 1.9 or 6.5 mg·kg^{-1}, Figure 2A–D) and male (0.65, 1.9 or 6.5 mg·kg^{-1}, Figure 3A–C) guinea pigs. However, ibodutant was effective in inhibiting the visceral hyperalgesia induced by TNBS both in female and male animals, although a gender-related difference in ibodutant efficacy was observed. In female animals, pretreated with TNBS, ibodutant at a low dose (0.33 mg·kg^{-1}) inhibited by 50% the abdominal contractions and completely inhibited the TNBS-induced visceral hyperalgesia at a higher dose (0.65 mg·kg^{-1}) (Figure 2). At this latter dose, ibodutant was ineffective when administered to male animals, while much higher doses (1.9 and 6.5 mg·kg^{-1}) did completely reverse the increase in abdominal contractions induced by TNBS (Figure 3).

Pharmacokinetic profile of ibodutant

The pharmacokinetic profile of ibodutant was assessed in guinea pigs following s.c. administration of two different drug doses. The time course of ibodutant present in the plasma and measured after 5, 15, 30 min, 1, 2, 4, 8, 24 and 48 h from its dosing did not differ between male and female animals, both at the lower (0.73 mg·kg^{-1}) and the higher (2.1 mg·kg^{-1}) dose tested (Figure 4), and ibodutant at both doses rapidly achieved a peak plasma concentration. Table 1 summarizes the C_{max} and AUC_{0-t} values that showed no differences between female and male animals.

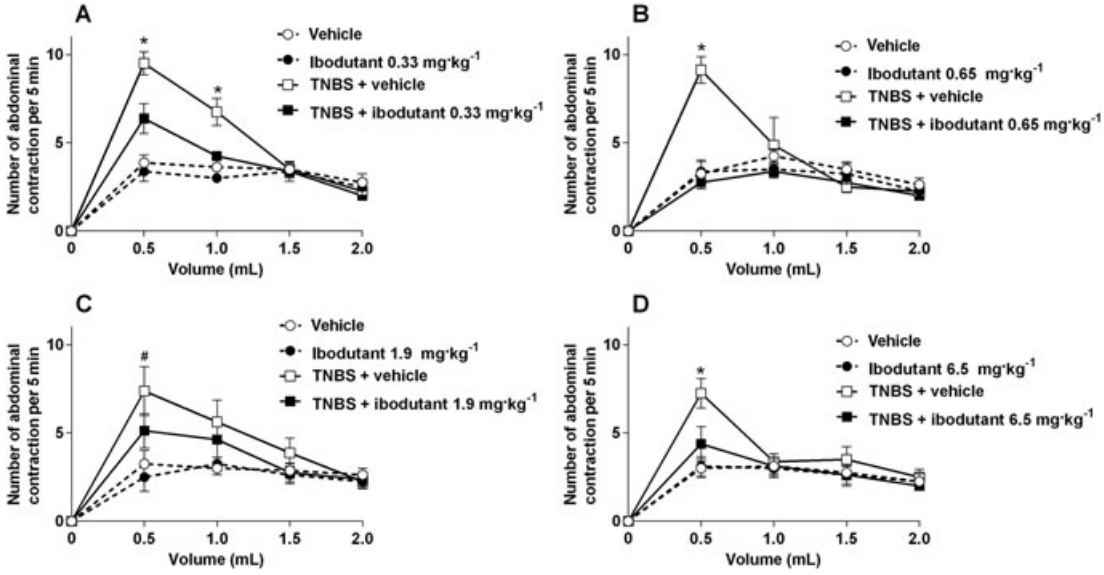

Figure 2

Effect of ibodutant or its vehicle s.c. administered 30 min before CRD in control or TNBS-treated female guinea pigs. Data are expressed as number of abdominal contraction measured in 5 min periods. Data points are the mean ± SEM of eight animals in each group. Data were analysed by two-way ANOVA followed by Bonferroni's multiple comparison test. *$P < 0.05$ TNBS + vehicle versus vehicle, ibodutant and TNBS; #$P<0.05$ TNBS + vehicle versus vehicle and ibodutant groups or TNBS + vehicle versus vehicle, ibodutant and TNBS + ibodutant (panels B and C).

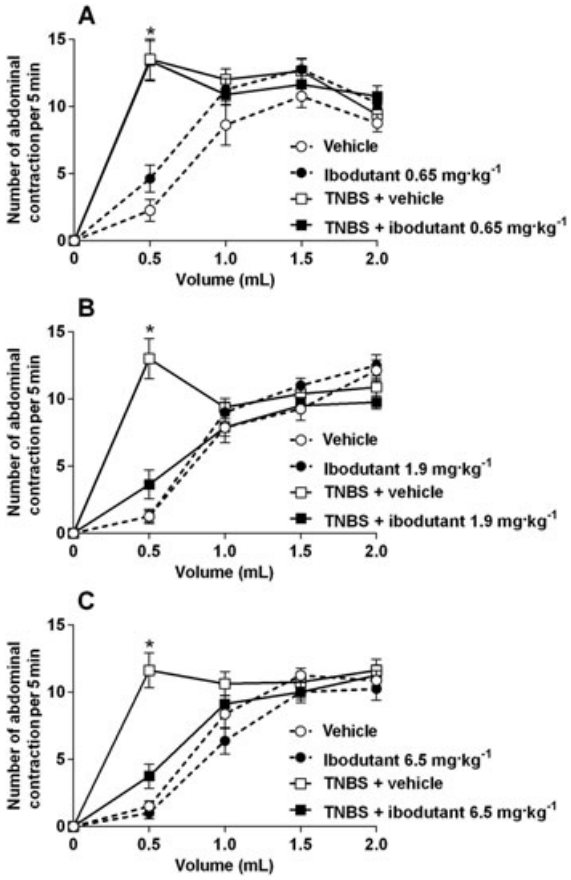

Figure 3

Effect of ibodutant or its vehicle s.c. administered 30 min before CRD in control or TNBS-treated male guinea pigs. Data are expressed as number of abdominal contraction measured in 5 min periods. Data points are the mean ± SEM of eight animals in each group. Data were analysed by two-way ANOVA followed by Bonferroni's multiple comparison test. *$P < 0.05$ TNBS + vehicle and TNBS + ibodutant versus vehicle and ibodutant (panel A) or TNBS + vehicle versus vehicle, ibodutant, and TNBS + ibodutant (panels B and C).

Release of NKA and SP tachykinins from distal colon

In a further series of experiments, the release of NKA and SP was determined in smooth muscle and mucosal preparations from distal colon of both female and male guinea pigs, which had been treated 3 days before with TNBS or saline (control). When tissues were stimulated with KCl (80 mM), the release of NKA from smooth muscle tissues was significantly greater in preparations from TNBS-treated female guinea pigs, whereas no corresponding differences were observed in males (Figure 5A). A significantly greater release of NKA occurred in male mucosal samples, compared with female samples, in control animals (Figure 5C). A slightly higher release of NKA in control female preparations, both smooth muscle and mucosal, following stimulation with capsaicin (10 μM) was also observed (Figure 5B and D), and two-way ANOVA analysis of data from mucosal tissues indicated a significant reduction after TNBS treatment ($F(1,12) = 5.101$, $P<0.05$).

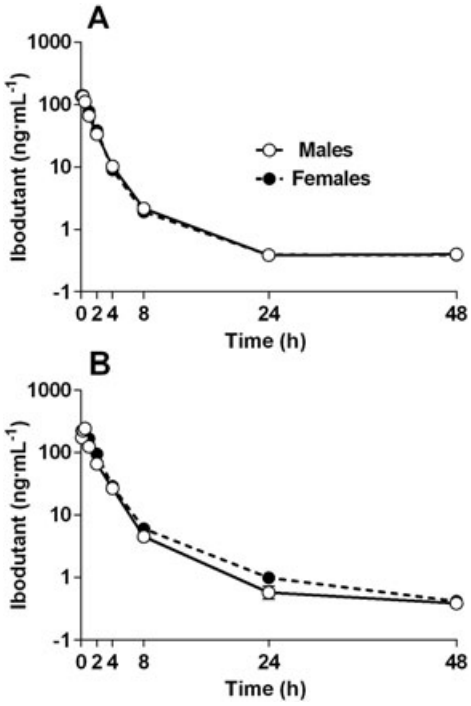

Figure 4

Plasma concentration-time course of ibodutant after s.c. administration to male and female guinea pigs. Each point represents the mean ± SEM of four animals plotted in a log-linear scale: Blood was collected from the jugular vein of guinea pigs at 0, 5, 15, 30 min, 1, 2, 4, 8, 24 and 48 h after dosing with ibodutant: 0.73 mg·kg^{-1} (A) or 2.1 mg·kg^{-1} (B).

Table 1

Key pharmacokinetic parameters of ibodutant following s.c. administration in male and female guinea pigs.

	C_{max} (ng·mL^{-1})	AUC_{0-t} (ng·h·mL^{-1})
Ibodutant 0.73 mg·kg^{-1}		
Males	150 ± 29.6	234 ± 54.2
Females	155 ± 11.1	238 ± 48.8
Ibodutant 2.1 mg·kg^{-1}		
Males	265± 47.2	535 ± 131
Females	234 ± 40.2	560 ± 83.0

Data represent the mean ± SD of single parameters calculated for each animal (n = 4 each group).

In the same samples, the content of SP was also detected, and the quantity following KCl or capsaicin in samples of both tissues was generally lower than that of NKA. On the other hand, as observed for NKA release, KCl induced a greater release of SP from smooth muscle preparation from TNBS-treated female guinea pigs (Figure 6A). Moreover, an analogous pattern to NKA data was observed in terms of greater KCl-induced release of SP from male, compared with

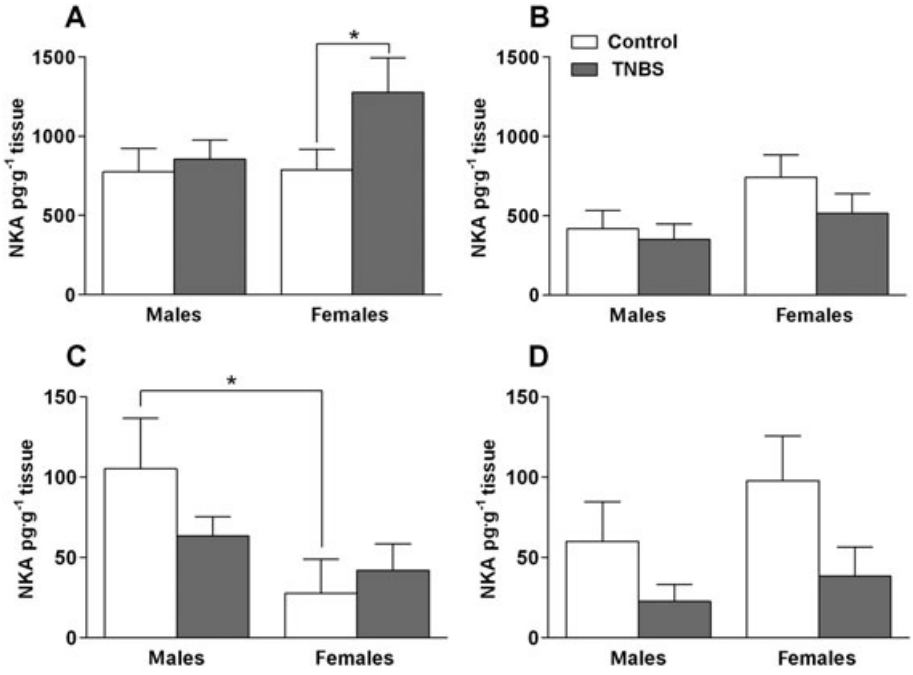

Figure 5

Released NKA in muscle and mucosal specimens from guinea pig distal colon. Effect of stimulation with KCl (80 mM) (A and C) or capsaicin (10 μM) (B and D) on the NKA release from muscle (A and B) and mucosal (C and D) tissue preparations from control and TNBS-treated male and female guinea pigs. Data are expressed as assessed by EIA method normalized to the tissue wet weight. Data points are the mean ± SEM of four to six animals in each group. Data were analysed by two-way ANOVA followed by Fisher's LSD *t*-test.

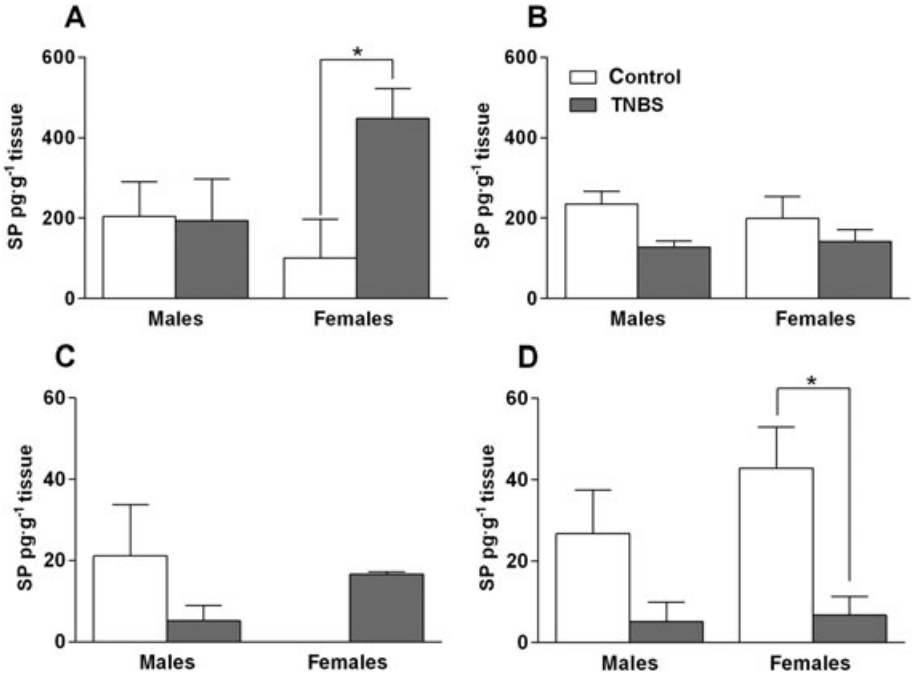

Figure 6

Released SP in muscle and mucosal specimens from guinea pig distal colon. Effect of stimulation with KCl (80 mM) (A and C) or capsaicin (10 μM) (B and D) on the SP release from muscle (A and B) and mucosal (C and D) tissue preparations from control and TNBS-treated male and female guinea pigs. Data are expressed as SP assessed by EIA, normalized to the tissue wet weight. Data points are the mean ± SEM of four to six animals in each group. Data were analysed by two-way ANOVA followed by Fisher's LSD *t*-test.

female control mucosal preparations (SP release was not detectable in control female preparations, Figure 6C), but also of a higher release following capsaicin stimulation, which was higher in mucosal preparations from control female guinea pigs (Figure 6D).

Discussion

Gender differences in pharmacotherapy for IBS have been already found, for instance for the 5-HT_3 antagonists alosetron (IBS-D) (Viramontes *et al.,* 2001) and the chloride channel activator lubiprostone (IBS-C) (Lunsford and Harris, 2010), but no mechanistic explanations have been provided. On the other hand, gender-related differences have been recorded at the level of the CNS, in terms of microstructural reorganization following pain or different metabolism of neuromodulators in IBS patients (Nakai *et al.,* 2005; Ellingson *et al.,* 2013; Labus *et al.,* 2013).

Our findings clearly show a gender-related difference between male and female guinea pigs with regard to peripheral mechanisms linked to activation of enteric neurons, both in control conditions and after induction of colitis. In a previous study by Kamp *et al.* (2003), visceral hyperalgesia to CRD was investigated in different strains of mice, and also in male and female animals belonging to the same strain (129S6 mice). Indeed, when the AUC values were compared, the responses of females were higher than those obtained with males, but no other parameters were recorded (Kamp *et al.*, 2003). In our experiments, we first found a gender-related different sensitivity to CRD in guinea pigs, both in control and after induction of TNBS colitis, but we cannot exclude the possibility that this difference can match or depend on a different inflammatory profile, which remains to be investigated. Interestingly, the greater hypersensitivity to CRD we measured with male guinea pigs nicely reflects the greater abdominal pressure recorded by electrical activity in healthy men as compared with women during inflation of a rectal balloon with increasing volumes (Sun and Read, 1989).

Intriguingly, the present results indicate that the tachykinin NK_2 receptor antagonist ibodutant in TNBS-treated guinea pigs inhibited the colonic hypersensitivity at lower doses than in male animals, thus reflecting results observed in the Phase II clinical study, in which ibodutant was more effective in women than men affected by IBS-D (Corsetti *et al.,* 2015). The colitis model induced by intrarectal administration of TNBS is characterized by inflammation and visceral hypersensitivity and thus reproduces some of the complex pathophysiology features of IBS (Del Valle-Pinero *et al.,* 2015). On the other hand, it is likely that the greater efficacy of ibodutant observed in the female animals may indeed depend on their lower colonic hypersensibility as compared with males.

A peripheral site of action of ibodutant seems more likely because previous studies established a peripheral site of action of NK_2 receptor antagonists in correcting visceral hyperalgesia (Laird *et al.,* 2001) and because of poor penetration of the blood brain barrier has been reported for this molecule (Menarini Ricerche, data on the file). The difference observed in ibodutant potency in relieving TNBS-induced visceral hyperalgesia cannot be explained by pharmacokinetic effects because identical plasma levels of the drug were recorded in male and female guinea pigs.

SP and NKA are expressed in not only extrinsic primary afferent neurones and differerent kind of myenteric intrinsic neurones, but also in enterochromaffin and immune cells or smooth muscle cells (see Lecci *et al.,* 2004). In the present experiments, higher levels of NKA and SP following stimulation by KCl were released by enteric neurons from samples of male guinea pig colon mucosa, indicating that nerve terminals and neurons of this tissue layer in males are able to release higher levels of tachykinins than the remainder. Indeed, NK_2 receptors have been previously identified in colon muscularis mucosae, of both human and guinea pig (Gates *et al.,* 1988; Renzi *et al.,* 2000; Warner *et al.,* 2000; Kamikawa *et al.,* 2002), and NKA appears as one of the more important spasmogens when compared with other neurotransmitters, such as ACh or 5-HT (Kamikawa *et al.,* 2002). Interestingly, in a comparative study performed in colonic mucosal tissue from IBS male and female patients and controls, the expression of the mRNA encoding for SP and NKA formation (TAC1) and for the tachykinin NK_1 and NK_2 receptors (TACR1 and TACR2, respectively) was investigated, and an higher expression of tachykinin receptors was demonstrated in control, compared with female IBS patients, whereas no differences were observed in men (Chang *et al.,* 2012). Taken together, our present and the previous results agree with the importance of mucosal nerves activation in IBS (Buhner *et al.,* 2012; Dothel *et al.,* 2015).

The treatment with capsaicin 10 μM is assumed to release tachykinins from primary afferents, although previous studies have shown that the bulk of tachykinins in the gastrointestinal tracts originates from enteric neurons and not from primary afferents (Holzer, 1991; Lippi *et al.,* 1998). The fact that releasable NKA and SP are lower in mucosal specimens from TNBS-treated animals is more likely to be attributed to an exaggerated release during TNBS colitis. Similarly, the higher levels of NKA and SP released from smooth muscle samples in response to KCl in TNBS-treated female guinea pigs, compared with controls, would suggests a lower release during colitis and deserves further investigation.

Overall, the results obtained in the current study highlights gender-related differences in colonic hypersensibility and mucosal nerve activation in a preclinical model of colitis.

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Author contributions

C.A.M. conceived the study; L.B. and P.S. designed the experiments; L. B. conducted visceral hyperalgesia experiments; V.D. and A.C. conducted and analysed pharmacokinetic experiments; R.B. set up the LC-MS analytical assay; P.S. performed *in vivo* treatments and release experiments; M.T. performed

peptide extraction; F.B., performed quantitative EIA assays; S.M. analysed data and draft the manuscript; C.A.M., S.M. wrote the manuscript and critically revised the manuscript for important intellectual content. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of pre-clinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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